were combined and concentrated to dryness *in vacuo*. The residue was dissolved in 2 ml. of methanol and added dropwise with stirring to 50 ml. of ether. The resulting precipitate was washed three times with ether and dried.

Anal. Calcd. for $C_{15}H_{28}N_2O_{10}$: C, 45.45; H, 7.12; N, 7.07; N-methyl, 3.79. Found: C, 45.77, 45.27; H, 7.63, 7.55; N, 6.92, 7.13; N-methyl, 3.93.

p-(p-Hydroxyphenylazo)-benzenesulfonic Acid Salt of Hygromycin B.—To 100 mg. of hygromycin B in 0.5 ml. of water was added 200 mg. of *p*-(*p*-hydroxyphenylazo)-benzenesulfonic acid in 2 ml. of water. The solution was cooled to 5°. After one hour the precipitate that formed was removed by filtration and was recrystallized three times from water. The yield was 124 mg. The salt gradually decomposed above 210°. An unexplained inconsistency in the carbon analyses of a number of salt preparations prevents the unequivocal assignment of a formula. However, one of the following two formulas appears to be correct: $C_{39}H_{48}N_6S_2O_{18}$ or $C_{39}H_{48}N_6S_2O_{17}$. Analytical values for two preparations are shown.

Anal. Calcd. for $C_{39}H_{48}N_6S_2O_{18}$: C, 49.15; H, 5.08; N, 8.82; S, 6.73. Calcd. for $C_{39}H_{48}N_6S_2O_{17}$: C, 49.99; H, 5.16; N, 8.97; S, 6.84. Found: C, 49.36, 50.13; H, 4.60, 5.06; N, 8.20, 8.69; S, 6.48, 6.67.

INDIANAPOLIS 6, INDIANA

[CONTRIBUTION NO. 2276 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The α-Chymotrypsin-catalyzed Hydrolysis of α-N-Benzoyl-β-(4-pyridyl-1-oxide)-L-alanine Methyl Ester and of α-N-(Nicotinyl-1-oxide)-L-phenylalanine Methyl Ester[1]

BY ROBERT L. BIXLER AND CARL NIEMANN[2]

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Consideration of the consequences of multifunctionality of the catalytically active site of α-chymotrypsin and of a class of its representative specific substrates led to the conclusion that under comparable conditions the rate of the α-chymotrypsin-catalyzed hydrolysis of α-N-(nicotinyl-1-oxide)-L-phenylalanine methyl ester should be substantially greater than that of α-N-benzoyl-β-(4-pyridyl-1-oxide)-L-alanine methyl ester. This prediction has been verified by the observation that the relative rate of hydrolysis of the former specific substrate is *ca.* 200 times greater than that of the latter.

If it is assumed that the catalytically active site of α-chymotrypsin and its representative specific substrates, derived from α-amino acids, are multifunctional,[3-8] we may represent the specific substrate by the notation $R_1CHR_2R_3$, where R_1 is the acylamino moiety, R_2 the α-amino acid side chain and R_3 the group capable of undergoing reaction, and the quasi complementary loci of the catalytically active site by the symbols, ρ_1, ρ_2 and ρ_3. With such a representation and when the nature of R_3 is invariant, we may postulate that with all other reaction parameters held constant the rate of formation of reaction products will be dependent upon the frequency with which interactions of the type R_1-ρ_1, R_2-ρ_2 and R_3-ρ_3 are consummated, recognizing that the occurrence of such events will be dependent upon the degree to which $R_1 \neq R_2 \neq R_3$ and the relative magnitude of the various R-ρ interactions.[4-8] The correlative postulate is that R-ρ interactions other than those of the R_1-ρ_1, R_2-ρ_2 and R_3-ρ_3 type will be essentially non-productive and competitive.

When the R_2-ρ_2 interaction is dominant and when $R_1 \neq R_2 \neq R_3$, it would be expected that the probability of productive modes of combination would be greater than when $R_1 = R_2 \neq R_3$, because in the latter case interactions of the type R_2-ρ_1 and R_1-ρ_2 would tend to occur with greater frequency and thus increase the probability of unproductive and competitive modes of combination. A more extreme case would arise when fragments of the groups $R_1 \neq R_2$ are interchanged to produce a pair of specific substrates of the type $R'CONHCH(CH_2R'')CR_3$ and $R''CONHCH(CH_2R')CR_3$, where R_3 is invariant and where the nature of R′ and R″ are such as to minimize steric differences and to maximize differences in binding energies with respect to interaction with the ρ_1 and ρ_2 loci so that the order of affinity for the ρ_2 locus is R″ > R′. From the argument given above it would be expected that in the presence of α-chymotrypsin the first specific substrate would be hydrolyzed at a substantially faster rate than the second with all other factors held constant.

Earlier studies[9,10] had shown that the interaction of the catalytically active site of α-chymotrypsin with benzamide was considerably more favorable than its interaction with nicotinamide, presumably because of a greater extent of solvation of the latter species.[11] This knowledge coupled with that with respect to the so-called side chain specificity of α-chymotrypsin[3] prompted the intended comparison of the behavior of α-N-nicotinyl-L-phenylalanine methyl ester with that of α-N-benzoyl-β-(4-pyridyl)-L-alanine methyl ester[12] in the presence of α-chymotrypsin with the expectation that the former specific substrate would be hydrolyzed more rapidly than the latter.

When it was found that β-(4-pyridyl)-DL-alanine was not very soluble in water,[13] it was decided

(1) Supported in part by a grant from the National Institutes of Health, U. S. Public Health Service.

(2) To whom inquiries regarding this article should be sent.

(3) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(4) H. T. Huang and C. Niemann, THIS JOURNAL, **74**, 4634, 5963 (1952).

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(8) S. Levine, *Enzymologia*, **16**, 256, 265 (1953–1954).

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(11) H. T. Huang and C. Niemann, *ibid.*, **74**, 101 (1952).

(12) This pair was chosen rather than the pair α-N-isonicotinyl-L-phenylalanine methyl ester and α-N-benzoyl-β-(4-pyridyl)-L-alanine methyl ester, arising from the simple interchange of the groups R′ and R″, because of a desire to maintain more nearly constant distances between the pyridine nitrogen atoms and the asymmetric carbon atoms of the members of the pair.

(13) R. L. Bixler and C. Niemann, *J. Org. Chem.*, in press.

to compare the behavior of the pair α-N-(nicotinyl-1-oxide)-L-phenylalanine methyl ester and α-N-benzoyl-β-(4-pyridyl-1-oxide)-L-alanine methyl ester since it was likely that both of these compounds would be reasonably soluble in water.

The synthesis of α-N-benzoyl-β-(4-pyridyl-1-oxide)-L-alanine methyl ester has been described.[13] In considering methods for the preparation of α-N-(nicotinyl-1-oxide)-L-phenylalanine methyl ester, it was hoped that a method of acylation, similar to those based upon the use of nicotinyl chloride or azide,[14,15] could be devised for the direct acylation of L-phenylalanine methyl ester. Since it was anticipated that nicotinyl chloride-1-oxide would not be stable, because of interaction between the N-oxide and acid chloride groups, attention was directed to the preparation of nicotinyl and isonicotinyl azide 1-oxide. Methyl isonicotinate-1-oxide was prepared by the method of Ochiai,[16] and converted to the hydrazide. Reaction of the hydrazide with nitrous acid gave only isonicotinic acid-1-oxide, presumably *via* the azide, which being soluble in water hydrolyzed to give the acid. The same results were obtained with nicotinic acid hydrazide-1-oxide.

The usefulness of cyanomethyl esters as acylating agents in other situations[17] led us to prepare the cyanomethyl ester of nicotinic acid-1-oxide. However, under the conditions ordinarily used[17] the above ester did not react with L-phenylalanine methyl ester.

Attempts to oxidize α-N-nicotinyl-DL-phenylalanine methyl ester with hydrogen peroxide in glacial acetic acid gave only oils. However, the oxidation of α-N-nicotinyl-DL-phenylalanine to α-N-(nicotinyl-1-oxide)-DL-phenylalanine was successful largely because the greater water solubility of the latter compound relative to the starting material permitted the ready isolation of the desired product. Esterification of α-N-(nicotinyl-1-oxide)-DL-phenylalanine with methanol and thionyl chloride[18] gave α-N-(nicotinyl-1-oxide)-DL-phenylalanine methyl ester. Enzymatic hydrolysis of the DL-ester with α-chymotrypsin gave α-N-(nicotinyl-1-oxide)-L-phenylalanine and α-N-(nicotinyl-1-oxide)-D-phenylalanine methyl ester. Esterification of the L-acid, with methanol and thionyl chloride,[18] gave α-N-(nicotinyl-1-oxide)-L-phenylalanine methyl ester. This latter ester also was prepared by esterification of L-phenylalanine, acylation of the ester with nicotinyl chloride, enzymatic hydrolysis to α-N-nicotinyl-L-phenylalanine, oxidation and finally esterification.

It has been noted previously[19] that kinetic studies involving the use of α-N-acylated aromatic α-amino acid esters present experimental difficulties, created by the requirement for relatively low enzyme concentrations, that still require solution. Since the problem at hand was to compare the relative rates of hydrolysis of α-N-benzoyl-β-(4-pyridyl-1-oxide)-L-alanine methyl ester and of α-N-(nicotinyl-1-oxide)-L-phenylalanine methyl ester, in order to confirm or disprove the prediction that the former compound would be hydrolyzed at a substantially slower rate in the presence of α-chymotrypsin, no attempt was made to evaluate K_S and k_3 for the above two systems, because it was anticipated that both would require the use of relatively low enzyme concentrations. However, it was clear that a procedure was required that would yield a reasonable basis of comparison of these two specific substrates.

In principle two types of procedures are available. In the first, a relatively high value for $[S]_0$ is chosen, *i.e.*, *ca.* 10^{-1} M for the case at hand, so that $[S]_0 > K_S$ and $v_0 \doteq k_3[E]$. While specific substrate concentrations of the order of 10^{-1} M were obtainable with α-N-benzoyl-β-(4-pyridyl-1-oxide)-L-alanine methyl ester, they were not obtainable with α-N-(nicotinyl-1-oxide)-L-phenylalanine methyl ester.

In the second procedure, a relatively low value for $[S]_0$ is chosen so that $[S]_0 < K_S$ and $v_0 \doteq k_3[E]\cdot[S]_0/K_S$. The conditions of the second procedure were approximated, as closely as was experimentally possible, by examining the kinetics of the α-chymotrypsin catalyzed hydrolysis of α-N-benzoyl-β-(4-pyridyl-1-oxide)-L-alanine methyl ester and of α-N-(nicotinyl-1-oxide)-L-phenylalanine methyl ester at values of $[S]_0$ of 1.15 and 1.10×10^{-4} M, respectively. In order to avoid differential losses of enzyme to the surfaces of volumetric equipment,[19,20] the same enzyme solution was used for both experiments and at a concentration of 2.32×10^{-4} mg. protein-nitrogen per ml. or *ca.* 6.6×10^{-8} M, based upon an assumed molecular weight of monomeric α-chymotrypsin of 22,000 and a nitrogen content of 16.0%. This concentration was the minimum at which the hydrolysis of α-N-benzoyl-β-(4-pyridyl-1-oxide)-L-alanine methyl ester could be followed with a *p*H-Stat[21] and the maximum at which the hydrolysis of α-N-(nicotinyl-1-oxide)-L-phenylalanine methyl ester could still be similarly observed. The results of these experiments are summarized in Table I.

It will be noted that the relationship between extent of reaction *vs.* time was essentially linear for the former specific substrate and was non-linear for the latter. While the assumption that $v_0 = k_3[E][S]_0/K_S$ demands that the above relationship be linear in both cases, it must be remembered that with the former specific substrate the total extent of reaction was but 3.9%, whereas with the latter it was 80%. With an extent of reaction of the latter magnitude it is not unreasonable to expect that inhibition of the reaction by its reaction by its reaction products could lead to a significant departure from linearity. Thus, as a reasonable approximation, we may assume that with both specific substrates $v_0 \doteq k_3[E][S]_0/K_S$.

The value of v_0 for the α-chymotrypsin-catalyzed hydrolysis of α-N-(nicotinyl-1-oxide)-L-phenylala-

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TABLE I

COMPARISON OF THE RATES OF THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF α-N-BENZOYL-β-(4-PYRIDYL-1-OXIDE)-L-ALANINE METHYL ESTER (I) AND α-N-(NICOTINYL-1-OXIDE)-L-PHENYLALANINE METHYL ESTER (II)[a]

I		II	
$[S]_0 = 1.15 \times 10^{-4}$ M		$[S]_0 = 1.10 \times 10^{-4}$ M	
$[NaOH]^b = 1.162 \times 10^{-3}$ M		$[NaOH]^b = 4.65 \times 10^{-3}$ M	
$[E]^c = 2.32 \times 10^{-4}$ mg. P-N/ml.		$[E]^c = 2.32 \times 10^{-4}$ mg. P-N/ml.	
[NaCl] = 0.02 M		[NaCl] = 0.02 M	
t^d	Scale reading[e]	t^d	Scale reading[e]
0	0	0	0
2	4.5	0.4	40.0
4	9.0	0.8	71.5
6	13.5	1.2	95.0
8	17.5	1.6	115.0
10	21.5	2.0	132.5
12	25.5	2.4	148.0
14	29.5	2.8	160.5
16	33.5	3.2	172.0
v_0^f	2.65×10^{-7}	v_0^f	5.43×10^{-5}
$v_0/[E][S]_0^g$	9.9	$v_0/[E][S]_0^g$	2130
% Reaction	3.9	% Reaction	80

[a] In aqueous solutions at 25° and *p*H 7.90 ± 0.01. [b] Concn. of base employed in *p*H-Stat. [c] Apparent enzyme concentration. [d] In min. [e] In units of 10^{-3} ml. of base. [f] In units of *M*/min., calculated as before[21] by the empirical orthogonal polynomial procedure of Booman and Niemann. [g] In units of ml./min. mg. protein-nitrogen per ml.

nine methyl ester was found to be *ca.* 200 times greater than that for the comparable hydrolysis of α-N-benzoyl-β-(4-pyridyl-1-oxide)-L-alanine methyl ester. If, in both cases, $v_0 \doteq k_3[E][S]_0/K_S$, it follows that the values of k_3/K_S for the two specific substrates differ similarly in magnitude. Since it is unlikely that the values of K_S for these two specific substrates will differ by a factor significantly greater than 10, particularly in view of the observation that the inhibition of the α-chymotrypsin catalyzed hydrolysis of α-N-benzoyl-L-valine methyl ester by α-N-benzoyl-β-(4-pyridyl-1-oxide)-L-alaninate under comparable conditions led to a value of $K_I = 20 \times 10^{-3}$ *M*, it can be inferred that the greater rate of the α-chymotrypsin-catalyzed hydrolysis α-N-(nicotinyl-1-oxide)-L-phenylalanine methyl ester relative to that of α-N-benzoyl-β-(4-pyridyl-1-oxide)-L-alanine methyl ester can be ascribed to a larger value of k_3 for the former specific substrate. Thus, accepting the validity of the proceding assumptions, the prediction made earlier has been verified and the hypothesis upon which it was based has received experimental support.

Experimental[22,23]

α-N-Nicotinyl-DL-phenylalanine.—To a solution of 22 g. (0.0775 mole) of α-N-nicotinyl-DL-phenylalanine methyl ester[14] in 500 ml. of methanol was added 39 ml. of 2 *N* sodium hydroxide (0.0775 mole) and the solution warmed for 1 hr. at 50°. The methanol was evaporated, the residue dissolved in water and acidified to *p*H 4.3. An oil formed which crystallized slowly to give, after recrystallization from water, 20.0 g. (95%) of acid, m.p. 198–199° dec., lit.[24] 198°.

α-N-(Nicotinyl-1-oxide)-DL-phenylalanine.—Twenty grams of the above acid was dissolved in 75 ml. of glacial acetic acid and 17 ml. of 30% hydrogen peroxide at 90°. The solution was held at 90° for 16 hr., cooled, diluted with 150 ml. of water and filtered. The precipitate was air-dried, to give 15.0 g. (71%) of product, m.p. 230° with dec. This solid was suspended in 400 ml. of 0.5 *N* hydrochloric acid, filtered and the residue recrystallized from 1.5 l. of water to give 12.0 g. (57%) of the desired acid, m.p. 232.0–232.5° with dec.

Anal. Calcd. for $C_{15}H_{14}O_4N_2$ (286): C, 62.9; H, 4.9; N, 9.8. Found: C, 63.0; H, 5.0; N, 9.8.

α-N-(Nicotinyl-1-oxide)-DL-phenylalanine Methyl Ester. The ester was prepared *via* a thionyl chloride esterification[18] of the parent acid in 93% yield. The ester was obtained as an oil by the addition of pentane to a concentrated chloroform solution. The oil was crystallized by trituration with water. Recrystallization from water gave a product, m.p. 158.5–160° with dec.

Anal. Calcd. for $C_{16}H_{16}O_4N_2$ (300): C, 64.0; H, 5.4; N, 9.3. Found: C, 64.1; H, 5.3; N, 9.5.

α-N-(Nicotinyl-1-oxide)-L-phenylalanine.—To a suspension of 8.0 g. of the above ester in 200 ml. of water was added 40 mg. of α-chymotrypsin and the *p*H of the solution maintained between 7–8 by the addition of 1 *N* sodium hydroxide. The asymmetric hydrolysis was completed in 20 minutes. The resultant clear solution (the solubility of the ester in water is about 2 g./100 ml.) was acidified to *p*H 2.0 with 12 *N* hydrochloric acid and allowed to stand for 30 minutes. The crystalline precipitate which had formed was recovered and recrystallized from 200 ml. of water to give 2.8 g. (73%) of L-acid, m.p. 218° with dec., $[\alpha]^{25}D$ −83.5° (*c* 1.6% in dimethylformamide).

Anal. Calcd. for $C_{15}H_{14}O_4N_2$ (286): C, 62.9; H, 4.9; N, 9.8. Found: C, 63.0; H, 4.9; N, 9.9.

This product was identical with that prepared by the hydrogen peroxide–acetic acid oxidation of α-N-nicotinyl-L-phenylalanine.

α-N-(Nicotinyl-1-oxide)-L-phenylalanine Methyl Ester.—Esterification of the L-acid as described for the DL-acid gave, after recrystallization from water, 74% of the L-ester, m.p. 161.0–161.2°, $[\alpha]^{25}D$ −57.7° (*c* 1.7% in methanol).

Anal. Calcd. for $C_{16}H_{16}O_4N_2$ (300): C, 64.0; H, 5.4; N, 9.3. Found: C, 63.8; H, 5.6; N, 9.3.

α-N-(Nicotinyl-1-oxide)-D-phenylalanine Methyl Ester.—The acidic filtrate resulting from the resolution of the DL-mixture, *vide post*, was neutralized to *p*H 7 and saturated with salt. An oil formed which, upon standing in contact with the solution for two days, crystallized in the form of needles. The crystalline D-ester was recovered, washed with water and dried *in vacuo* to give 2.8 g. (70%) of product, m.p. 160–161°, $[\alpha]^{25}D$ +58.2° (*c* 2.1% in methanol).

Anal. Calcd. for $C_{16}H_{16}O_4N_2$ (300): C, 64.0; H, 5.4; N, 9.3. Found: C, 63.8; H, 5.2; N, 9.2.

α-N-Nicotinyl-L-phenylalanine.—To a solution prepared by the addition of 16.3 ml. (0.112 mole) of triethylamine to a suspension of 24.3 g. (0.112 mole) of L-phenylalanine methyl ester hydrochloride[24] in 150 ml. of dry chloroform, was added a solution prepared from 20.0 g. (0.112 mole) of nicotinyl chloride hydrochloride and 31.2 ml. (0.224 mole) of triethylamine in 250 ml. of dry chloroform. The temperature of the reaction mixture rose to 50°, and the red solution was allowed to stand for 90 minutes, after which time it turned a pale yellow color. The chloroform solution was extracted with two 150-ml. portions of water, 75 ml. of saturated aqueous sodium bicarbonate, followed by 75 ml. of water, dried over magnesium sulfate and the solvent removed *in vacuo*. The residual oil was partially dissolved in 100 ml. of 30% aqueous-methanol, 50 mg. of α-chymotrypsin added and the *p*H of the solution maintained between *p*H 7–8 by the addition of 1 *N* sodium hydroxide. The methanol was removed in a stream of air and the solution acidified to *p*H 4.5. The resulting precipitate was recrystallized from water to give 27.2 g. (90%) of acid, m.p. 176–177° dec., lit.[15] 177–178°.

Oxidation of this acid with hydrogen peroxide–acetic acid and then esterification, essentially as described for the DL-compound, gave α-N-(nicotinyl-1-oxide)-L-phenylalanine methyl ester with properties identical with those given above.

Methyl Isonicotinate-1-oxide.—This ester was prepared by the hydrogen peroxide oxidation of methyl isonicotinate according to Ochiai.[16] The product was recrystallized from

(22) All melting points are corrected.

(23) Microanalyses by Dr. A. Elek.

(24) E. Wolf and A. M. Seligman, THIS JOURNAL, **73**, 2086 (1951).

a mixture of 60–70° ligroin and chloroform to give 67% of colorless needles, m.p. 121.0–121.5°.

Anal. Calcd. for $C_7H_7O_3N$ (153): C, 54.9; H, 4.6. Found: C, 54.9; H, 4.6.

Isonicotinyl Hydrazide-1-Oxide.—Reaction of the above ester with a twofold excess of hydrazine hydrate in warm methanol gave a 90% yield of the desired hydrazide, m.p. 229° dec., lit.[25,28] 227° dec., 233–234°.

Cyanomethyl Nicotinate-1-oxide.—Nicotinic acid-1-oxide was prepared by the method of Ochiai[16] to give 75% of the acid, m.p. 250–251°, lit.[27] 249°. To a suspension of 10 g. (0.072 mole) of this acid in 100 ml. of dry ethyl acetate was added 15 ml. (0.108 mole) of triethylamine and 8.16 g. (0.108 mole) of chloroacetonitrile. The gummy mixture formed on the addition of triethylamine dissolved upon the addition of the chloroacetonitrile. The resultant clear yellow solution was held at reflux for 90 minutes with stirring in order to break up the precipitate which formed. The stirred suspension was cooled in an ice-bath, filtered and the residue washed with 10 ml. of ethyl acetate. The residue was air dried, then suspended in 85 ml. of cold absolute methanol to remove the triethylamine hydrochloride, filtered, washed with 25 ml. of cold methanol and dried *in vacuo*. The product was recrystallized from ethyl acetate to give 10.0 g. (78%) of ester, m.p. 160–161°.

Anal. Calcd. for $C_8H_6O_3N_2$ (178): C, 53.9; H, 3.4; N, 15.7. Found: C, 53.7, 53.9; H, 3.6, 3.4; N, 15.8, 15.7.

Enzyme Experiments.—The general method has been described previously.[21] Other pertinent details are summarized in Table I. In addition to the experiments described in Table I the value of K_I for α-N-benzoyl-β-(4-pyridyl-1-oxide)-L-alaninate was evaluated against α-N-benzoyl-L-valine methyl ester with $[S]_0 = 2.04 \times 10^{-3}$ *M*, $[I] = 0.877 \times 10^{-3}$ *M*, [E] = 0.15 mg. protein-nitrogen per ml. and [NaCl] = 0.02 *M*. The value of v_0 obtained was 2.86×10^{-5} *M*/min. and the value of $[S]_0[E]/v_0$, 10.7 min./mg. protein-nitrogen per ml. Since for the same values of $[S]_0$, [E] and [NaCl], but with [I] = 0, $v_0 = 2.96 \times 10^{-5}$ *M*/min. and $[S]_0[E]/v_0$ = 10.3 min./mg. protein-nitrogen per ml., it may be inferred that K_I for α-N-benzoyl-β-(4-pyridyl-1-oxide)-L-alaninate is 20×10^{-3} *M* at *p*H 7.9 and 25°.

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[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, UNIVERSITY OF CALIFORNIA SCHOOL OF MEDICINE]

Citrovorum Factor Cyclodehydrase[1]

BY JAMES M. PETERS AND DAVID M. GREENBERG

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An enzyme has been separated from sheep liver acetone powders which converts citrovorum factor in the presence of adenosine triphosphate to a compound with an absorption spectrum similar to, but not identical with, that of $N^{5,10}$-methenyltetrahydrofolic acid. The spectrum of the enzymatically-formed product has its absorption maximum at 343 mμ, instead of at 354 mμ, which is the absorption maximum of $N^{5,10}$-methenyltetrahydrofolic acid. The enzymatically-formed material also differs from $N^{5,10}$-methenyltetrahydrofolic acid by being stable at *p*H 6.8. It is not readily decomposed by oxygen. The enzyme has its optimum activity at about *p*H 4 and requires a free sulfhydryl group for activity. The Michaelis constant of the enzyme for citrovorum factor was determined to be 1.7×10^{-6} *M*. The residual protein fraction, after extraction of the citrovorum factor cyclodehydrase, was found to act synergistically with citrovorum factor cyclodehydrase in converting citrovorum factor to a compound which could not be converted to $N^{5,10}$-methenyltetrahydrofolic acid by acidification.

Introduction

Recently we reported the partial purification and certain properties of a multi-enzyme system of sheep liver concerned with the conversion of citrovorum factor (N^5-formyltetrahydrofolic acid) to a serine aldolase cofactor.[2] Subsequent investigations have enabled us to separate an enzyme from sheep liver acetone powders which converts citrovorum factor to a compound with an absorption spectrum similar to that of anhydrocitrovorum factor (anhydroleucovorin, $N^{5,10}$-methyltetrahydrofolic acid). The enzyme was prepared by passing an aqueous extract of the acetone powder through an hydroxylapatite column (the aqueous eluate contained the enzyme) or by adsorption of the aqueous acetone powder extract on calcium phosphate gel (gel:protein ratio 1.2 or more; protein concentration 1% or less) followed by centrifugation of the gel (the supernatant fluid contained the

TABLE I

SUMMARY OF PURIFICATION OF SHEEP LIVER CITROVORUM FACTOR CYCLODEHYDRASE

Fraction	Mg. protein per ml.	Apparent enzyme units[a] per ml.	Apparent specific activity
Aqueous extract of whole liver	22	0	0
Aqueous extract of acetone powder	24	30	1
Calcium phosphate gel fraction[b]	2	32	16
40–60% satd. $(NH_4)_2SO_4$ fraction	105	2015	19
Calcium phosphate gel fraction of 45–55% $(NH_4)_2SO_4$ fraction	2	47	24
Calcium phosphate gel fraction after treatment with CM-cellulose[c]	1	72	72

[a] One unit of citrovorum factor cyclodehydrase is defined as that amount of protein which will bring about a change in the optical density at 343 mμ of 0.001 unit in 30 minutes when incubated with 0.2 μmole leucovorin, 2 μmoles ATP, 2 μmoles $MgSO_4$ and 100 μmoles of sodium citrate buffer, *p*H 6.0, in 3.0 ml. at 25°. [b] Calcium phosphate gel fraction = eluate from aqueous extract of acetone powder and calcium phosphate gel (gel:protein ratio = 1.2 or more; protein concentration 1% or less). [c] The calcium phosphate gel fraction was negatively adsorbed at *p*H 5.4 on carboxymethyl-cellulose.[3]

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(2) J. M. Peters and D. M. Greenberg, *J. Biol. Chem.*, **226**, 329 (1957).